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Review article

# Transient expression assays in grapevine: a step towards genetic improvement

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## Summary

In the past few years, the usefulness of transient expression assays has continuously increased for the characterization of unknown gene function and metabolic pathways. In grapevine (*Vitis vinifera* L.), one of the most economically important fruit crops in the world, recent systematic sequencing projects produced many gene data sets that require detailed analysis. Due to their rapid nature, transient expression assays are well suited for large-scale genetic studies. Although genes and metabolic pathways of any species can be analysed by transient expression in model plants, a need for homologous systems has emerged to avoid the misinterpretation of results due to a foreign genetic background. Over the last 10 years, various protocols have thus been developed to apply this powerful technology to grapevine. Using cell suspension cultures, somatic embryos, leaves or whole plantlets, transient expression assays enabled the study of the function, regulation and subcellular localization of genes involved in specific metabolic pathways such as the biosynthesis of phenylpropanoids. Disease resistance genes that could be used for marker-assisted selection in conventional breeding or for stable transformation of elite cultivars have also been characterized. Additionally, transient expression assays have proved useful for shaping new tools for grapevine genetic improvement: synthetic promoters, silencing constructs, minimal linear cassettes or viral vectors. This review provides an update on the different tools (DNA constructs, reporter genes, vectors) and methods (*Agrobacterium*-mediated and direct gene transfer methods) available for transient gene expression in grapevine. The most representative results published thus far are then described.

## Introduction

Transient expression assays provide a rapid and convenient tool for basic research in plant biology. They have been developed for gene function studies (Hellens *et al.*, 2005; Lee and Yang, 2006) and have also proved helpful for assessing the activity of gene constructs before undertaking stable transformation (Sparkes *et al.*, 2006). Recently, many sequencing data sets have been released within the grapevine community, prompting research in the development of efficient transient expression systems in this species.

Grapevine (*Vitis vinifera* L.) is one of the most economically important fruit crops of the world, and it is widely cultivated for fruits, juice and especially for wine. Its genetic improvement relies on conventional breeding and genetic engineering, depending on the availability of germplasm resources and the identification of agronomically important genes (Burger *et al.*, 2009; Reisch *et al.*, 2012). The completion of the grapevine genome sequence project 7 years ago has opened the door to in-depth genetic studies (Jaillon *et al.*, 2007; Velasco *et al.*, 2007). Very recently, Di Genova *et al.* (2014) sequenced a table grape cultivar and compared it to the reference genome of the genotype PN40024 (Jaillon *et al.*, 2007), leading to the identification of 240 novel genes, as well as numerous structural variants and SNPs. In addition, transcriptome analyses were performed by RNA-seq (Venturini *et al.*, 2013; Zenoni *et al.*, 2010) and small RNAs libraries were obtained (Carra *et al.*, 2009; Han *et al.*, 2014; Mica *et al.*, 2010; Pantaleo *et al.*, 2010; Wang *et al.*, 2011). This genetic information could be exploited to identify genes or elucidate pathways involved in traits of agronomic importance (Di Gaspero and Cattonaro, 2010).

Genome annotation gives indications of the role of newly discovered genes. It is, however, insufficient to fully characterize their function and regulation. A gene's function can be investigated by knocking out or knocking down its expression. In the absence of mutant collections, which is the case for grapevine, RNA interference (RNAi) methods can be used. Since the pioneer experiments of Ecker and Davis (1986), efficient methods have been reported for disrupting gene expression through RNAi in plants (Huang *et al.*, 2012; Mc Ginnis, 2010; Ossowski *et al.*, 2008; Small, 2007). Overexpression or misexpression of a wild-type gene can also cause abnormal phenotypes, allowing the identification of pathway components undetected by loss-of-function analysis (Prelich, 2012).

Gene transfer technologies offer the opportunity to express exogenous sequences in target plant tissues and to interfere with endogenous genetic expression. These are therefore well suited for characterizing the function and regulation of newly discovered genes. Stable transformation allows the study of stable gene expression at the whole plant level. This approach has proved useful for functional studies in herbaceous model plants such as *Arabidopsis thaliana* and *Nicotiana benthamiana*, due to

easy regeneration of stable transformants (Goodin *et al.*, 2008; Koornneef and Meinke, 2010). However, stable transformation remains a long and random process and is unsuited to large-scale analyses, especially in grapevine. Despite the tremendous progress made in the last decade, it remains difficult to generate stably transformed whole grapevine plants (Vidal *et al.*, 2010). Alternatively, *Agrobacterium rhizogenes*-transformed roots (hairy roots) provide an interesting system for functional studies (Hu and Du, 2006). In grapevine, Gomez *et al.* (2009) produced hairy roots to localize anthocyanin transporter candidates (AM1 and AM3) to the tonoplast. Using the same system, ectopic expression of *VvMYBPA1* or *VvMYBPA2* provided clues on their roles in the regulation of the proanthocyanidin (PA) pathway (Terrier *et al.*, 2009). Likewise, Höll *et al.* (2013) recently demonstrated the role of *VvMYB15* in the synthesis of glycosylated stilbenes.

Transient expression assays provide the most efficient way to study many genes in a very short time. They are based on temporary, high-level transcription of DNA sequences that do not necessarily integrate into the plant genome. Methods for transient gene expression in plants were developed concurrently with stable transformation protocols in the 1980s. These mainly involve *Agrobacterium tumefaciens*-mediated transformation or direct gene transfer by chemical (polyethylene glycol, i.e. PEG, treatment) or physical (particle bombardment) techniques. Indeed, during a short period immediately following the cultivation with *A. tumefaciens*, many copies of the transgene are actively transcribed in the plant cells, allowing an expression up to 1000-fold higher than in stably transformed tissues (Janssen and Gardner, 1989). Likewise, direct transformation methods lead to rapid and high-level expression of the introduced DNA. In addition to transformation methods, inoculation of viral vectors is an efficient way to transfer exogenous DNA into plant cells (Scholthof *et al.*, 1996).

Leaf agro-infiltration represents a major historic breakthrough in transient expression assays. This method was first developed in *Nicotiana sp* (Schöb *et al.*, 1997; Scofield *et al.*, 1996; Tang *et al.*, 1996; Yang *et al.*, 2000) and in tomato, *Arabidopsis thaliana* and a few other species (Van der Hoorn *et al.*, 2000). It is based on the forced infiltration of *A. tumefaciens* into the intercellular spaces of the leaf parenchyma, using a needleless syringe or a vacuum pump. This method is easy and rapid, and significantly cheaper than most other methods for transient gene expression. It is therefore adapted for high-throughput studies. More recently, a simple agro-drenching method has been developed to deliver foreign DNA into plant cells. It consists of applying an *Agrobacterium* suspension in the immediate vicinity of plant roots. Reported for the first time in 2004, it allowed the inoculation of *N. benthamiana* and some other *Solanaceae* species with recombinant viral vectors (Ryu *et al.*, 2004).

In transient expression assays, protoplasts, cell suspension cultures, isolated organs or whole plants are subjected to the gene transfer process. Nonphotosynthetic tissues, such as onion epidermal cells or petals, are well suited for localization or quantitative expression studies involving fluorescence- or colour-based reporter genes (Scott *et al.*, 1999; Shang *et al.*, 2007; Yasmin and Debener, 2010).

Due to the current lack of mutant collections in grapevine, transient expression assays constitute an appropriate approach to decipher the huge amount of genetic information becoming available. Heterologous systems can be used and have proved helpful, as illustrated in recent reports. For example, agro-infiltration of leaves of *N. benthamiana* highlighted the role of the grapevine enzyme anthocyanin O-methyltransferase (AOMT), as well as its localization in the cytosol (Hugueney *et al.*, 2009). Using the same transient expression system, functional characterization of several stilbene synthase genes was achieved (Parage *et al.*, 2012). Likewise, the ATP-binding cassette protein ABCC1 was localized to the tonoplast (Francisco *et al.*, 2013). Particle bombardment of onion cells can also help investigate the localization of grapevine proteins, as shown for the zinc transporter ZIP3 in the plasma membrane (Gainza-Cortés *et al.*, 2012). However, gene expression in heterologous systems may exhibit aberrant traits, presumably due to a foreign genetic background. Grapevine is a woody perennial species, characterized by unique features whose study preferentially requires a homologous gene transfer system (Vidal *et al.*, 2010).

Over the last 10 years, efforts have been made to apply a wide variety of transient expression assays to grapevine, involving *Agrobacterium*-mediated or direct transformation protocols (Tables 1 and 2). In addition, although mechanical inoculation of viruses to grapevine plants is rarely successful, specific viral vectors have been developed for high-level and systemic expression of exogenous DNA. After an overview of the different tools and methods available for transient gene expression in grapevine, this review focuses on representative published results. Emphasis has been placed on *V. vinifera* which is the most common grapevine species cultivated in the world.

## DNA constructs

### Various constructs for different purposes

Various DNA constructs may be designed for the study of plant gene function and regulation. Overexpression and knock-down experiments are very helpful in learning about a gene of unknown function. Molecular dissection of the promoter-proximal region of a gene contributes to identifying important cis-regulatory elements. It is also possible to construct tools for the validation of new promoters and silencing constructs (Figure 1).

#### *Gene overexpression*

In gain-of-function experiments, the gene of interest is fused to a strong promoter, to observe the effects of ectopic overexpression (Figure 1a). The function of a gene can be deduced from the phenotypic changes associated with its overexpression, such as alteration of metabolic pathways or increased/decreased tolerance to biotic or abiotic stresses. Overexpression requires the fusion of the sequence of interest with a strong constitutive promoter such as the cauliflower mosaic virus 35S (CaMV35S) promoter commonly used for plant transformation (Hull *et al.*, 2000). The main disadvantage of this approach is that the gene product is synthesized in excessive amounts, possibly in tissues where it is not usually present.

#### *Gene silencing by RNAi*

In knock-down or loss-of-function experiments, the effector sequence is the same as an endogenous gene, to induce gene silencing through RNAi (Figure 1b). Expression of the cloned sequence generates double-stranded-(ds-)RNAs that cause the specific degradation of homologous mRNAs in transformed tissues. As in overexpression experiments, the effector sequence is fused to a strong constitutive promoter to significantly reduce the expression of the target gene. Disruption of gene function can be obtained by overexpressing a homologous sense (cosuppression) or an antisense sequence, as discovered by Waterhouse

**Table 1** Summary of transient expression assays in grapevine (*Vitis vinifera* L.) using *Agrobacterium*-mediated transformation

| Method | Plant tissue | Cultivar | *Agrobacterium tumefaciens* strain | Application | Studied genes/ sequences | Pathway | Reporters | References |
|---|---|---|---|---|---|---|---|---|
| Agro-infiltration (syringe) | Attached leaves of *in vitro* plantlets | Aleatico, Moscato Giallo, Sugraone | AGL1, GV3101, LBA4404 | Technical focus | – | – | GFP, RFP, YFP | Zottini *et al.* (2008) |
| | | Superior seedless | AGL1, GV3101 | Gene silencing | hpRNA against *VvPDS* | – | GFP (transformation), *VvPDS* (silencing) | Urso *et al.* (2013) |
| Agro-infiltration (vacuum) | Attached leaves of *in vitro* plantlets | Cabernet Franc, Syrah, Zinfandel | EHA105 | Viral vector engineering | GLRaV-2 cDNA | – | GFP (infection), *VvPDS* and *VvChl1* (silencing) | Kurth *et al.* (2012) |
| | | Thompson seedless | C58C1 (pCH32) | Overexpression | D4E1 (synthetic AMP) | Defence (*A. vitis, X. ampelinus*) | – | Visser *et al.* (2012) |
| | Detached leaves of *in vitro* plantlets | Cabernet Franc | GV3101 | Gene silencing | hpRNA against *VvPGIP1* | Defence (*B. cinerea*) | GUS | Bertazzon *et al.* (2012) |
| | | Cabernet Sauvignon, Cinsault, Muscat Ottonel, Syrah | C58C1 (pCH32) | Overexpression | *VvVST1* | Defence (*P. viticola*) | GFP, GUS | Santos-Rosa *et al.* (2008) |
| | | Carignane | GV3101 | Overexpression | *VpGLOX* | Defence (*E. necator*) | GUS | Guan *et al.* (2011) |
| | | | | | *VpPR10.2* | Defence (*P. viticola*) | GUS | He *et al.* (2013) |
| | | | | Promoter analysis | *VpSTS* | Defence (*E. necator*) | GUS | Xu *et al.* (2010) |
| | | | LBA4404 | Overexpression | *VpPR10.1* | Defence (*P. viticola*) | – | Xu *et al.* (2014) |
| | | Syrah | C58C1 (pCH32) | Overexpression | *VvNPR1* | Defence (*P. viticola*) | – | Le Henanff *et al.* (2009) |
| | Leaves | Grenache | GV2260 | Viral vector engineering | GLRaV-2 cDNA | – | GFP, GUS (infection) | Liu *et al.* (2009) |
| Agro-drenching | Roots of *in vitro* plantlets | Prime | C58C1 (pCH32) | Viral vector engineering | GVA cDNA | – | GFP (infection), *VvPDS* (silencing) | Muruganantham *et al.* (2009) |
| | | Prime, Thompson seedless | EHA105 | Viral vector engineering | GRSPaV cDNA | – | GFP (infection) | Meng *et al.* (2013) |
| Cocultivation | Cell suspension culture | Gamay Red | EHA105 | Promoter analysis | *VvDFR* | Flavonoids | GUS | Gollop *et al.* (2002) |
| | Somatic embryos | Chardonnay | GV3101 | Ami-RNA validation (cotransformation) | amiRNAs against *Grapevine fanleaf virus* and GUS sensor | Defence (*GFLV*) | GUS | Jelly *et al.* (2012) |
| | | Thompson seedless | EHA105 | New promoters testing | CaMV35S, CsVMV, *Arabidopsis* ACT2 promoters | – | GFP | Li *et al.* (2001) |
| | | | | | BDDPs with CaMV35S, CsVMV promoters and enhancers | – | GFP, GUS | Li *et al.* (2004) |
| | | | | | | | *VvMybA1* | Li *et al.* (2011) |
| | | | | | 31 grapevine promoters (PR1, PAL, Ubiquitin etc.) | - | GUS, *VvMybA1* | Li *et al.* (2012) |

 

**Table 2** Summary of transient expression assays in grapevine (*Vitis vinifera* L.) using direct transformation methods

| Method | Plant tissue | Cultivar | Application | Studied genes/ sequences | Pathway | Reporter | References |
|---|---|---|---|---|---|---|---|
| Biolistics | Cell suspension culture | Cabernet Sauvignon | Promoter analysis | *VvAdh1, VvAdh2* | Abiotic stress | Luc/GUS | Torregrosa *et al.* (2002) |
| | | | | *VvAdh2* | Abiotic stress | Luc/GUS | Verriès *et al.* (2004) |
| | | Chardonnay | Protein–DNA nteraction (cotransformation) | *VvMYBA1, -F1, -PA1, -PA2* and *VvCHS1, VvCHS2, VvCHS3* promoters | Flavonoids | Dual Luc | Harris *et al.* (2013) |
| | | | | *VvMYB5a,-5b* and *VvANR, VvANS,VvCHI, VvF3′5′H, VvLAR1* promoters | Flavonoids | Dual Luc | Deluc *et al.* (2008) |
| | | | | *VvMYBA1, -A2* and *VvUFGT* promoter | Flavonoids | GFP, dual Luc | Walker *et al.* (2007) |
| | | | | *VvMYBF1* and *VvANR, VvCHI, VvFLS1, VvLDOX* promoters | Flavonoids | Dual Luc | Czemmel *et al.* (2009) |
| | | | | *VvMYBPA1* and *VvANR, VvCHI, VvF3′5′H, VvLAR1, VvLDOX* promoters | Flavonoids | Dual Luc | Bogs *et al.* (2007) |
| | | | | *VvMYC1* and *VvMYB5a, -5b, -A1, -A2, -PA1* and *VvANR, VvCHI, VvMYC1, VvUFGT* promoters | Flavonoids | Dual Luc | Hichri *et al.* (2010) |
| | | Chardonnay Pinot Noir | Protein–DNA interaction (cotransformation) | *VvMYB14, -15* and *VvSTS29, -41* promoters | Stilbenes | Dual Luc | Höll *et al.* (2013) |
| | Leaf sections | Chardonnay | Promoter analysis | *VvPGIP1* promoter | Defence (*B. cinerea*) | GUS | Joubert *et al.* (2013) |
| | Somatic embryos | Thompson seedless | Promoter analysis | *VvPGIP1* promoter | Defence (*B. cinerea*) | GUS | Joubert *et al.* (2013) |
| PEG treatment | Protoplasts | Cabernet Sauvignon | Promoter analysis | *VvMSA* | Abiotic stress | Dual Luc | Saumonneau *et al.* (2012) |
| | | | Protein–DNA interaction (cotransformation) | *VvWRKY1* and *VvJAZ1.1, VvLOX* promoters | JA defence pathway | Dual Luc | Marchive *et al.* (2013) |
| | | | Protein localization | *VvMYC1* | Flavonoids | YFP | Hichri *et al.* (2010) |
| | | | Protein–protein interaction and localization (cotransformation) | *VvMSA* | Abiotic stress | YFP | Saumonneau *et al.* (2008) |

*et al.* (1998). However, hairpin constructs comprising two self-complementary sequences separated by a short loop sequence silence their target with a greater efficiency (Smith *et al.*, 2000). Additionally, artificial microRNAs (amiRNAs) produced by modified miRNA precursors have proved effective for silencing endogenous genes (Schwab *et al.*, 2006).

### *Screening of amiRNA silencing constructs*

Transgenic plants expressing amiRNAs designed to target exogenous sequences such as viral genomes can be protected against virus attacks (Niu *et al.*, 2006). Before undertaking stable transformation, the efficiency of amiRNA constructs can be assessed *in vivo* by transient expression assays (Duan *et al.*, 2008; Parizotto *et al.*, 2004). Interference between amiRNAs and a desired viral target is easily studied by performing cotransformation assays involving the amiRNA precursor construct together with a gene-silencing reporter construct which comprises a reporter gene fused to the target sequence (Figure 1c). In case of correct processing of the amiRNA precursor and recognition of the target sequence by the amiRNAs, silencing of the RNAi reporter can occur through specific cleavage.

### *Study of regulatory elements*

For promoter sequence analyses and transcriptional studies, or subcellular localization of gene products, various cis-acting regulatory elements are fused to a reporter gene (Figure 1d). A native promoter can be fused to a reporter gene to assay transcriptional activity under varied environmental conditions, as well as in different types of tissue. Moreover, the fusion product of such a chimerical gene can be easily localized to a cellular compartment. Promoter deletion analysis involves a series of constructs comprising varying parts of a promoter region fused to a reporter gene. This allows the identification of specific regulatory elements in proximal regions of a gene. Fusion constructs can also be used to study protein–DNA interactions. Interactions between regulatory proteins and gene promoters can be visualized by cotransformation assays. The first construct contains a sequence that codes for the regulatory protein, while

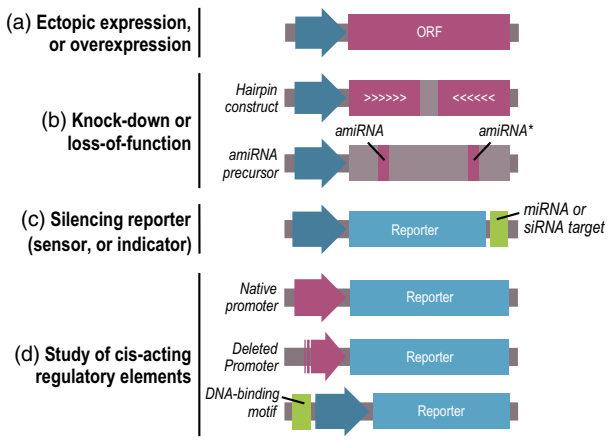


**Figure 1** DNA constructs designed for the study of gene function and regulation and for validation of silencing constructs. Arrows represent promoter sequences. Sequences of interest are shown in red or green. amiRNA and amiRNA* indicate the guide and the passenger strands, respectively. (a) In overexpression studies the sequence of interest is fused to a strong promoter. (b) For knock-down experiments, the effector sequence is fused to a strong constitutive promoter; hairpin constructs and amiRNA precursors are highly efficient to induce gene silencing. (c) Functionality of amiRNAs can be assessed using a gene-silencing reporter construct which comprises a reporter gene fused to the target sequence. (d) For promoter sequence analyses and transcriptional studies, or subcellular localization of gene products, various cis-acting regulatory elements are fused to a reporter gene.

the second construct comprises the promoter of a candidate target sequence fused to a reporter gene. In addition, transient expression assays offer a unique opportunity to rapidly validate synthetic promoter constructs comprising the designed sequence fused to a reporter gene.

## Reporter genes

Reporter genes have long proved useful for distinguishing transformed from untransformed cell tissues and optimizing transformation protocols through expression quantification (Matthews *et al.*, 1995). They can be used to investigate the transcriptional activity of a particular gene under various environmental or physiological conditions and to localize gene products (Rosellini, 2012). Most common reporter genes are of bacterial or animal (invertebrates) origin (Matthews *et al.*, 1995). Additionally, to monitor gene silencing, various endogenous constitutive genes may be used as reporter genes.

### *Common reporter genes*

In grapevine, the uidA gene (*gus*), as well as bioluminescent (*luc*) and fluorescent (*gfp* and its derivatives and *rfp*) reporter genes, have long been used to assess the efficiency of transformation protocols (Vidal *et al.*, 2010). Recently, they allowed the development of methods for transient gene expression (Santos-Rosa *et al.*, 2008; Zottini *et al.*, 2008). Many other applications have been reported. For example, *luc* genes contributed to the study of promoter activity in Cabernet Sauvignon cell suspension (Torregrosa *et al.*, 2002) or protoplast cultures (Marchive *et al.*, 2013; Saumonneau *et al.*, 2012). New synthetic promoters were tested in Thompson seedless somatic embryos using *gus* and *gfp* (Li *et al.*, 2004 and Li *et al.*, 2011). To validate amiRNA constructs designed to control virus infection, a GUS-based RNAi reporter was used in somatic embryos of Chardonnay (Jelly *et al.*, 2012).

### *VvMYBA1*, a plant reporter gene

*MYBA* genes encode transcription factors (TFs) that control the anthocyanin biosynthetic pathway and have been proposed as reporter genes since the early 90s (Ludwig *et al.*, 1990). Indeed, anthocyanins accumulate to very high levels in transformed tissues and are visible to the naked eye. By expressing the grapevine *VvMYBA1* sequence in somatic embryos of the cultivar Thompson seedless, Li *et al.* (2011) observed visible anthocyanin accumulation and demonstrated the potential of this gene as a homologous reporter gene for transient expression assays. However, the use of *MYBA* genes is restricted to experiments for which anthocyanin coloration of tissues does not interfere with the expression of investigated genes or pathways.

### *Endogenous plant genes as silencing reporters*

The endogenous *phytoene desaturase* (*PDS*) gene and the *magnesium chelatase subunit* gene (*Chl1*) were both used as endogenous reporters to validate constructs inducing gene silencing in *N. benthamania* and tobacco plants (Golenberg *et al.*, 2009; Ruiz *et al.*, 1998). Inactivation of these genes results in loss of chlorophyll, leading to visible leaf bleaching. These reporter genes have been successfully used in grapevine to monitor gene silencing by hairpin constructs (Urso *et al.*, 2013) and to develop specific virus-induced gene silencing (VIGS) vectors (Kurth *et al.*, 2012; Muruganantham *et al.*, 2009).

### *Monitoring and quantification of reporter gene expression*

The detection of Luc or GUS activity requires enzymatic assays using rather expensive substrates. Specifically, GUS assays involve destructive histochemical staining of tissue for protein detection and localization. In contrast, the expression of fluorescent proteins is visualized directly by fluorescence microscopy. Even better, *MYBA* gene expression is visible to the naked eye and its detection does not require any sophisticated equipment. *MYBA* genes therefore constitute convenient and cost-effective reporters for gene transfer technologies in plants. Similarly, expression of internal silencing reporter genes like *PDS* and *Chl1* is readily visualized. Reporter gene expression can be localized within organs or cells and monitored under various conditions. The use of live-cell reporters allows a continuous control of expression.

Quantification of reporter gene expression is possible and has been performed in most studies. For *gfp*, the percentage of Thompson seedless somatic embryos expressing fluorescence could be determined, and a rating scale enabled evaluation of reporter expression levels (Li *et al.*, 2001). Similarly for *gus*, according to the intensity of blue stain of Chardonnay somatic embryos, a scale could be defined for evaluating the level of RNA silencing using a GUS-sensor construct (Jelly *et al.*, 2012). For *PDS*, the number of chlorotic spots on transformed leaves could be evaluated and quantitatively compared to untransformed controls (Urso *et al.*, 2013). Another sophisticated method based on computer analysis of digital images related to spectrophotometric measures of purified anthocyanin enabled estimation of *VvMYBA1* expression levels in Thompson seedless somatic embryos (Li *et al.*, 2011).

However, enzymatic assays allow more accurate quantification. In grapevine, quantitative measurement of GUS expression by this method has been applied to the study of synthetic promoters, for

example in Thompson seedless somatic embryos (Li *et al.*, 2004) and in leaves of the cultivar Carignane (Xu *et al.*, 2010). Likewise, Luc assays can give precise measures of reporter expression. These exploit firefly or *Renilla* luciferases, which use different substrates for generation of luminescence. The firefly Luc reporter has a relatively short half-life compared to fluorescent proteins and is thus recommended for precise analyses (Verriès *et al.*, 2004). Moreover, a dual luciferase assay developed for data normalization has been applied to transcriptional studies in cell suspension cultures of Chardonnay (Bogs *et al.*, 2007; Czemmel *et al.*, 2009; Deluc *et al.*, 2008; Harris *et al.*, 2013; Hichri *et al.*, 2010; Höll *et al.*, 2013; Walker *et al.*, 2007) and for promoter analysis in protoplasts of Cabernet Sauvignon (Marchive *et al.*, 2013).

### Vectors

Plasmid vectors are available for *A. tumefaciens*-mediated or direct transformation. In addition, plant viruses can be modified to deliver genes into plant tissues.

#### *Ti-plasmid-based vectors*

*Agrobacterium tumefaciens*-mediated transformation depends on *vir* genes and T-regions of a large native Ti-plasmid that can be engineered for biotechnological use (Păcurar *et al.*, 2011). The ability of *vir* genes to act in *trans* led to the development of binary vector systems that simplify plasmid manipulation (Hoekema *et al.*, 1983). Moreover, site-specific recombination-based cloning systems, such as the Gateway® technology, helped to overcome cloning difficulties due to a limited number of useful restriction sites in binary vectors (Nakagawa *et al.*, 2009). These efficient and reliable cloning systems are well suited for high-throughput analysis of plant genes. Moreover, a large set of Gateway®-compatible destination vectors are available for many applications (Karimi *et al.*, 2002; Murai, 2013). Binary vectors most often used for transient expression assays in grapevine are pBIN19 and derivative vectors (Le Henanff *et al.*, 2009; Li *et al.*, 2001 and Li *et al.*, 2004; Santos-Rosa *et al.*, 2008; Visser *et al.*, 2012), pCambia (He *et al.*, 2013; Xu *et al.*, 2010) and varied Gateway® destination vectors (Jelly *et al.*, 2012; Li *et al.*, 2011; Urso *et al.*, 2013). Xu *et al.* (2014) used a pER8 vector comprising an oestradiol-inducible promoter that significantly increased transgene expression.

#### *Direct transformation vectors and linear minimal cassettes*

Some original experiments that demonstrated the feasibility of direct transformation by protoplast electroporation involved Ti-plasmid vectors (Krens *et al.*, 1982; Langridge *et al.*, 1985). However, *E. coli*-based cloning vectors are convenient for direct transformation by protoplast permeation or particle bombardment. In grapevine, Kovalenko *et al.* (1997) observed that higher expression levels could be obtained with a linearized plasmid rather than with a circular vector. Later, the use of minimal gene cassettes, which are linear DNA fragments comprising the gene of interest flanked by regulatory sequences, was shown to be as effective as traditional circular plasmids in *V. vinifera* Chardonnay (Vidal *et al.*, 2006). Such minimal cassettes are highly desirable in stable transformation projects to avoid integration of vector backbone sequences (Fu *et al.*, 2000). In a transient expression assay, Sanjurjo *et al.* (2013) recently demonstrated the importance of protecting the 3′-end of the linear minimal cassette to attain DNA stability and efficient gene expression.

Up to now, transient expression assays using direct transformation involved varied vectors such as: pBI (Hébert *et al.*, 1993; Kikkert *et al.*, 1996; Vidal *et al.*, 2003), pSAN (Vidal *et al.*, 2003 and Vidal *et al.*, 2006), pLuc (Saumonneau *et al.*, 2012; Torregrosa *et al.*, 2002; Verriès *et al.*, 2004) or pCambia (Joubert *et al.*, 2013). In a series of cotransformation assays for studying protein–DNA interactions, a promoter-Luc fusion was carried on pLuc, while a TF gene was cloned into pART7 (Bogs *et al.*, 2007; Czemmel *et al.*, 2009; Harris *et al.*, 2013; Hichri *et al.*, 2010; Höll *et al.*, 2013; Walker *et al.*, 2007). A Gateway® vector was used for protein subcellular localization (Hichri *et al.*, 2010).

#### *Viral vectors*

Viral vectors can be engineered to express a sequence of interest or to induce VIGS (Becker and Lange, 2010; Scholthof *et al.*, 1996). Thus, VIGS has become a common reverse genetics tool for functional studies in model plants (Huang *et al.*, 2012). A need for viral vectors adapted to infect grapevine has recently emerged. Different grapevine viruses have been engineered to express exogenous DNA and could be agro-inoculated to several cultivars: the *Vitivirus Grapevine virus A* (GVA) (Muruganantham *et al.*, 2009), the *Closterovirus Grapevine leafroll-associated virus-2* (GLRaV-2) (Kurth *et al.*, 2012; Liu *et al.*, 2009) and the *Foveavirus Grapevine rupestris stem pitting-associated virus* (GRSPaV) (Meng *et al.*, 2013). As a proof of concept, these studies involved *gus* or *gfp* for assessing viral infection and the two internal reporters *PDS* and *Chl1* for assessing gene-silencing potential.

For a GLRaV-2-derived vector, systemic gene expression was detectable from 4 weeks postinoculation (Kurth *et al.*, 2012). It was established that expression occurred exclusively at the RNA level and was strikingly stable and persistent. Indeed, infected plants still expressed the exogenous sequence up to 15 months postinoculation. Moreover, the infection could be transmitted by grafting from inoculated plants to many important varieties. However, the use of viral vectors for functional analysis has not yet been reported.

## Methods for gene transfer

Methods for transient expression assays in grapevine involve *Agrobacterium tumefaciens*-mediated transformation, or chemical/physical methods for direct gene transfer to plant cells. Mechanical inoculation of viral vectors has not yet been reported in grapevine, so viral vectors are transferred by transformation methods.

### *Agrobacterium tumefaciens*-mediated gene transfer

*Agrobacterium tumefaciens*-mediated gene transfer methods were developed in grapevine in the early 1990s. Baribault *et al.* (1989) first succeeded in the transformation of cell suspension cultures of Cabernet Sauvignon. The *A. tumefaciens* strains most frequently used for transient expression assays in grapevine possess a C58 chromosomal background: GV3101 (pMP90) (Koncz and Schell, 1986), C58C1 (pCH32) (Hamilton *et al.*, 1996) and EHA105 (Hood *et al.*, 1993). C58C1 (pCH32) and EHA105 contain extra copies of *vir* genes that make them hypervirulent.

#### *Cocultivation*

Cocultivation with *A. tumefaciens* is the most common method to obtain stably transformed grapevines (Vidal *et al.*, 2010). This

also provides a straightforward method for transient expression assays. Up to now, these assays, based on short-term high-level expression of transgenes after cocultivation, mainly involved somatic embryos selected at the mid-cotyledonary stage of development of Thompson seedless (Li *et al.*, 2001, 2004, 2011, 2012) and Chardonnay (Jelly *et al.*, 2012). Naturally colourless embryos are particularly convenient for monitoring the expression of fluorescence- (Li *et al.*, 2001 & Li *et al.*, 2004) and colour-based reporter genes (Jelly *et al.*, 2012; Li *et al.*, 2011). Besides, Gollop *et al.* (2002) reported the cocultivation of cell suspension cultures of Gamay Red for studying the regulation of *dihydroflavonol 4-reductase* (*VvDFR*).

Li *et al.* (2006) showed that the level of transgene expression increases with the duration of cocultivation time: around 50% of embryos expressed the reporter gene after a 24 h-long cocultivation, and almost 100% after a 48 h-long cocultivation. For an exposure longer than 96 h, prevalence of gene reporter-expressing embryos dramatically decreased, and tissues showed browning and ceased to grow. Indeed, the major drawback of *Agrobacterium* use is the induction of plant tissue necrosis probably resulting from bacterial infection. Perl *et al.* (1996) suggested that the intensity of browning rather depends on plant genotype and culture protocol and medium than on bacterial strain. Plant tissue necrosis can be reduced by preculture on a medium containing active charcoal for a few days (Jelly *et al.*, 2012; Li *et al.*, 2006) or by reducing bacterial suspension concentration and preconditioning bacteria in the plant culture medium (Iocco *et al.*, 2001). Washing steps and culture of embryos on a filter paper after cocultivation, as well as addition of the antioxidant dithiothreitol (DTT) to the culture medium, have also been shown to help reducing tissue browning (Li *et al.*, 2006 and Li *et al.*, 2008; Perl *et al.*, 1996).

### *Agro-infiltration*

*Agrobacterium tumefaciens* can be infiltrated into plant leaves using two different methods. The first method involves a needleless syringe that can be filled with the bacterial suspension then pressed against the underside of a leaf to infiltrate the suspension through the stomata (Zottini *et al.*, 2008). This method is fast and simple, but tends to restrict gene expression to the infiltration zones. The second method consists of plunging leaves or whole plants into the bacterial suspension and then transiently applying a vacuum pressure to facilitate liquid penetration into the mesophyll cells. Contrary to the syringe infiltration method, vacuum infiltration allows gene expression in the whole leaf. It is possible to vacuum-infiltrate either detached leaves (Bertazzon *et al.*, 2012; Guan *et al.*, 2011; He *et al.*, 2013; Le Henanff *et al.*, 2009; Santos-Rosa *et al.*, 2008; Xu *et al.*, 2010 and Xu *et al.*, 2014) or nondetached leaves, that is whole plants (Kurth *et al.*, 2012; Visser *et al.*, 2012). Interestingly, an effect of leaf position on agro-infiltration efficiency has been shown. The first full expanded leaf displayed higher gene expression than the second leaf in 8- to 10-week-old plantlets (Santos-Rosa *et al.*, 2008). Agro-infiltration is usually performed on tissues of young plantlets grown *in vitro*, as greenhouse-grown plants have often been described as recalcitrant to this technique (Wroblewski *et al.*, 2005; Zottini *et al.*, 2008). However, Ben-Amar *et al.* (2013) established a protocol to agro-infiltrate leaves of greenhouse-grown plants, using a vacuum device. Although so far achieved with rootstock varieties and not *V. vinifera*, vacuum agro-infiltration of greenhouse-grown plants constitutes an interesting technical improvement. Indeed, this technique makes it possible to perform transient assays without aseptic tissue culture facilities, as with *N. benthamiana* or tobacco.

The success of agro-infiltration has been found to be cultivar dependent (Santos-Rosa *et al.*, 2008). However, infiltration-based assays could be developed for several economically important wine and table varieties (Table 1). Agro-infiltration was classically used for introducing gene constructs driven by a Ti-plasmid. Additionally though, this method has enabled the introduction of virus-derived vectors into several *V. vinifera* cultivars (Kurth *et al.*, 2012).

### *Agro-drenching*

Agro-drenching has been developed in grapevine for delivering an infectious viral cDNA clone of GVA that could not be inoculated using leaf agro-infiltration (Muruganantham *et al.*, 2009). This method has enabled the transfer of a silencing construct carried by a GVA-derived vector to be used in gene functional studies. Roots of young *in vitro* plantlets were slightly injured with a needle then immersed into a nutrient liquid medium containing the bacteria. Ten days were then required to achieve plantlet infection and recover viral molecules in the plant sap. The method developed in grapevine is slightly more complicated than the original soil drench method used with *Nicotiana* species (Ryu *et al.*, 2004). It has been validated for the inoculation of the grapevine cultivars Prime and Thompson seedless (Meng *et al.*, 2013; Muruganantham *et al.*, 2009).

## Direct gene transfer methods

Direct gene transfer (transfection) methods require permeation of protoplast membranes by a chemical (PEG) treatment or by electroporation to allow direct DNA uptake, or cell bombardment of plant tissues with high-velocity microparticles coated with the DNA of interest.

### *PEG treatment and electroporation of protoplasts*

Protoplast-based protocols for grapevine stable transformation are poorly developed, as culture and regeneration of *Vitis* spp protoplasts is hampered by the release of large amounts of polyphenols and phytoalexins in the culture medium (Commun *et al.*, 2003; Reustle and Natter, 1994). However, some success has been reported for the hybrid Seyval blanc (Reustle *et al.*, 1995) and an interesting method was developed in which whole plants of *V. vinifera* Koshusanjaku were regenerated from protoplasts through somatic embryogenesis (Zhu *et al.*, 1997).

Protoplast electroporation has generally been used for virus inoculation (Valat *et al.*, 2000 and Valat *et al.*, 2006). In the late 1990s, this method was proposed to investigate various *STS* promoters (Brehm *et al.*, 1999). However, recently reported transient expression assays involving protoplasts were based on chemical transformation. The use of PEG-treated protoplasts prepared from Cabernet Sauvignon cell suspension cultures has been reported for protein subcellular localization (Hichri *et al.*, 2010), for functional analysis of promoters (Saumonneau *et al.*, \2012) and for the study of protein/protein (Saumonneau *et al.*, 2008) or DNA/protein (Marchive *et al.*, 2013) interactions.

### *Particle bombardment (biolistics)*

Particle bombardment allows the manipulation of intact plant cells or organs of any plant species. In grapevine, biolistics was first developed for the transformation of the *Vitis* hybrid Chancellor (Hébert *et al.*, 1993; Kikkert *et al.*, 1996) and the *V. vinifera* cv. Chardonnay (Vidal *et al.*, 2003). This method was

also developed to inoculate the *Grapevine fanleaf virus* to hybrid rootstocks (Valat *et al.*, 2003). Although biolistics is mainly reported as a stable transformation method, it is also convenient for transient expression assays. The first application for functional studies was proposed by Torregrosa *et al.* (2002) and Verriès *et al.* (2004), who described the transformation of cell suspension cultures of Cabernet Sauvignon for analysing the promoter region of *alcohol dehydrogenase* (*Adh*) genes. Since then, cell suspension cultures of Chardonnay have largely been used for functional studies of genes involved in flavonoid synthesis (Bogs *et al.*, 2007; Czemmel *et al.*, 2009; Deluc *et al.*, 2008; Harris *et al.*, 2013; Hichri *et al.*, 2010; Höll *et al.*, 2013; Walker *et al.*, 2007). More recently, leaf sections of Chardonnay and somatic embryos of Thompson seedless were subjected to particle bombardment for studying the regulation of the *VvPGIP* defence gene (Joubert *et al.*, 2013).

Particle bombardment remains quite difficult to perform and requires the adjustment of a number of critical variables such as helium pressure, diameter of particles, cartridge preparation or distance from target plant material. Moreover, it is a costly method, due to the necessity of purchasing a biolistic device and expensive consumables. Particle bombardment presents some real advantages, however, such with no limitation on species range or genotype and simple plasmid construction. Moreover, biolistics allows for cotransformation with multiple genes (Vidal *et al.*, 2010).

## Applications of transient expression assays

The principal domains studied to date are the flavonoid pathway and the plant response to biotic and abiotic stresses. Besides, extensive studies of new promoters valuable for gene transfer were carried out.

### Elucidation of the flavonoid biosynthesis pathway

Flavonoids have received extended attention in grapevine, due to their contribution to wine colour, taste and health-promoting effects (Jimenez-Garcia *et al.*, 2013). They are also involved in the protection against many abiotic and biotic stresses (Brunetti *et al.*, 2013). These secondary metabolites comprise three major derivatives: flavonols, anthocyanins and proanthocyanidins (PAs). PAs, also called condensed tannins, and flavonols both play a role in the bitterness and astringency of wine. PAs have also been shown to be important defence molecules, and flavonols protect the inflorescences from UV damage (Czemmel *et al.*, 2012). Anthocyanins are reddish purple compounds that accumulate in the berry skin and thus contribute to the grape and wine colour. MYB TFs are key regulators of the flavonoid pathway (Czemmel *et al.*, 2012). Transient expression assays based on biolistic transfection of grapevine cell suspension cultures were essential for the investigation of these genetic regulators.

To identify target genes of MYB TFs in the biosynthesis of flavonoids, cotransformation studies involved an effector construct containing the *MYB* gene together with a reporter construct comprising the promoter of a candidate target gene fused to *luc* or *gus*. In this way, the grapevine TFs MYBA1 and MYBA2 were shown to specifically regulate the *UDP-glucose: flavonoid 3-O-glucosyltransferase* (*UFGT*) in anthocyanin biosynthesis (Walker *et al.*, 2007). Remarkably, this study showed that diverse mutations at the *VvMYBA* locus can be responsible for the absence of anthocyanins in white grape skin, as suggested by previous studies on orthologous genes in Kyoho, a cultivar of *V. labruscana* (Kobayashi *et al.*, 2002). With the same approach, MYBPA1 was shown to be involved in the regulation of PA biosynthesis but not anthocyanin (Bogs *et al.*, 2007), while MYB5a and MYB5b were shown to activate promoters of central pathway genes involved in flavonoid biosynthesis (Deluc *et al.*, 2008). In addition, the light-inducible TF MYBF1 was shown to specifically activate the expression of the flavonol synthase-encoding gene *VvFLS1*, as well as genes involved in the synthesis of chalcones and flavanones upstream of dihydroflavonols and other flavonoids (Czemmel *et al.*, 2009).

In these transient expression assays, MYB TFs (except MYBF1) activity required the presence of a basic helix-loop-helix (bHLH) TF encoded by a cotransferred *Arabidopsis* gene. Thereafter, Hichri *et al.* (2010) isolated a grapevine bHLH TF (called MYC1) that was shown, in biolistic cotransfection assays, to interact with MYB5, MYBA, as well as MYBPA TFs, to activate genes involved in the biosynthesis of anthocyanins and PAs. MYC1 is therefore an important part of the transcriptional cascade regulating the PAs and anthocynanins pathways of flavonoid biosynthesis in grapevine. In the same study, cellular localization and transport of MYC1 into the nucleus were examined by PEG-mediated transfection of protoplasts with a chimeric construct comprising the *MYC1* gene sequence fused to the *yfp* reporter gene.

More recently, Harris *et al.* (2013) studied the regulation by MYB TFs of three grapevine *chalcone synthase* (*CHS*) genes that catalyse the first committed step of the flavonoid pathway. Although all three genes seemed to be regulated by MYBPA1 and MYBPA2, consistent with a role in condensed tannin synthesis, differential responses to activation by other MYB TFs were observed. For example, the *VvCHS2* promoter was specifically activated by the light-inducible MYBF1 TF involved in flavonol synthesis.

In a former study, a transient expression assay based on cocultivation of Gamay Red cell suspension cultures with *A. tumefaciens* enabled the investigation of the promoter of the *DFR* gene involved in anthocyanin and PA synthesis and highlighted its light inducibility (Gollop *et al.*, 2002).

### Study of defence-related genes and sequences

*Vitis vinifera* is the main cultivated grape species, due to its superior organoleptic qualities. Unfortunately, it is highly susceptible to many microbial infections, particularly to fungal diseases such as powdery and downy mildew, caused by *Erisyphe necator* and *Plasmopara viticola*, respectively. These infections are often treated with phytochemicals suspected to cause adverse health and environmental effects. Consequently, many research programs focus on the improvement of grapevine resistance, either by hybridization with resistant grape species or by transgenesis. Wild grapes having long been subjected to natural selection generally present some rustic characteristics such as tolerance to biotic and abiotic stresses (Alleweldt and Possingham, 1988; Staudt and Kassemeyer, 1995). The exploitation of these natural genetic resources is facilitated by the availability of germplasm collections comprising many wild grape accessions (www.eu-vitis.de; www.ars-grin.gov/npgs). Transient expression assays can improve our understanding of basal defence mechanisms in grape species. They can also contribute to characterize genes that could be used in marker-assisted breeding, as well as to develop wild-derived or synthetic resistance genes to produce stably transformed elite cultivars.

### *Vitis vinifera* genes

The function of genes supposed to be involved in response to biotic stresses can be investigated by ectopic expression. Using vacuum agro-infiltration of leaves, overexpression of a *V. vinifera* gene encoding the key regulatory protein nonexpressor of pathogenesis-related 1 (NPR1) was shown to positively regulate *Pathogenesis-Related* (*PR*) genes upon *P. viticola* challenge, thus stimulating innate defences (Le Henanff *et al.*, 2009). Conversely, loss-of-function experiments can help determine to what extent endogenous levels of a protein contribute to plant defence. Efficient systems to transiently silence endogenous genes to evaluate their role in restricting pathogen attack were recently reported. Grapevine polygalacturonase-inhibiting protein1 (PGIP1) is a well-characterized cell-wall protein that is effective against *Botrytis cinerea* causing gray mould disease. A construct to express a double-stranded RNA homologous to *VvPGPIP1* was introduced into vacuum-infiltrated leaves and caused efficient silencing of its endogenous target (Bertazzon *et al.*, 2012). PGIP could then be extracted from agro-infiltrated leaves, and its inhibitory activity against purified *B. cinerea* polygalacturonase was determined *in vitro* and compared to that of unsilenced controls. Similarly, using a hairpin construct complementary to the endogenous reporter gene *PDS*, Urso *et al.* (2013) developed a gene-silencing system using leaf agro-infiltration. With the final goal of identifying genes involved in the resistance to powdery mildew in particular genotypes, they could show that agro-infiltration does not interfere with the development of *E. necator* on agro-infiltrated leaves, thereby allowing further gene-silencing experiments coupled with infection studies. However, as shown in tobacco (Pruss *et al.*, 2008), agro-infiltration itself can induce host defence responses that sometimes complicate or prevent the interpretation of results. No clear conclusions could thus be drawn from the infection by *P. viticola* of leaves transformed with a stilbene synthase (*STS*) gene, because agro-infiltration caused defence responses that interfered with the development of the pathogen (Santos-Rosa *et al.*, 2008).

The regulation of defence genes can be studied by transient expression of promoter fragments fused to a reporter gene. Promoter deletion analysis of *VvPGIP1* was performed by particle bombardment of grapevine leaf discs and somatic embryos, allowing the characterization of the core promoter as well as other cis-acting regulatory elements (Joubert *et al.*, 2013). In addition, cotransformation assays can be used to investigate hypothetical interactions between regulatory proteins and promoters of defence genes. In a co-transfection assay involving particle bombardment of cell suspension cultures, the grapevine TFs MYB14 and MYB15 were shown to regulate stilbene phytoalexin biosynthesis by specifically activating the promoters of *STS* genes (Höll *et al.*, 2013). Likewise, PEG-mediated cotransfection of protoplasts was performed to study the interaction of a grapevine WRKY TF with defence genes putatively involved in the jasmonic acid signalling pathway (Marchive *et al.*, 2013). In this study, WRKY1 was shown to activate the promoters of *VvLOX* and *VvJAZ1*, suggesting that these specific interactions could participate in increased tolerance to downy mildew of *VvWRKY1* overexpressing transgenic lines.

### *Wild grape resistance genes*

Unlike most *V. vinifera* cultivars, the Chinese *Vitis pseudoreticulata* accession Baihe-35-1 shows natural resistance against *E. necator* and *P. viticola* (Wan *et al.*, 2007). An *STS* gene responsible for the synthesis of resveratrol with antifungal properties was isolated from this wild accession, and promoter analysis was performed in the susceptible *V. vinifera* cv. Carignane (Xu *et al.*, 2010). By measuring the activity of *gus* fused to a series of *VpSTS* promoter deletion derivatives in agro-infiltrated leaves, the authors could characterize the regions important for the induction of the disease response. Other genes thought to be involved in the basal resistance of *V. pseudoreticulata* Baihe-35-1 were studied by ectopic expression in the susceptible cv. Carignane. Thus, enhanced resistance to *P. viticola* was induced by overexpression of the *pathogenesis-related* (*PR*) genes *VpPR10.1* (Xu *et al.*, 2014) and *VpPR10.2* (He *et al.*, 2013) in agro-infiltrated leaves. Similarly, overexpression of *VpGLOX* which encodes a glyoxal oxidase could inhibit *E. necator* hyphal development on transformed leaves (Guan *et al.*, 2011). Zhao *et al.* (2013) further showed evidence of $H_2O_2$ production in *VpGLOX* overexpressing *V. pseudoreticulata* which could explain the role of this gene in plant defence.

### *Synthetic antimicrobial compounds*

Broad spectrum antimicrobial compounds can be synthesized. Using an agro-infiltration assay involving whole plantlets, the synthetic peptide D4E1 was shown to have an inhibitory effect against the two grapevine-infecting bacterial pathogens *Agrobacterium vitis* and *Xylophilus ampelinus* (Visser *et al.*, 2012). This study is the first report of a promising prescreening procedure based on transient expression in *V. vinifera*.

### *amiRNAs targeting viruses*

Grapevine is affected by numerous viruses that compromise yield potential (Laimer *et al.*, 2009). The main approach to produce virus-resistant plants is based on RNA interference (RNAi) (Hamilton and Baulcombe, 1999). A method using modified miRNA precursor genes was recently developed to express artificial miRNAs (amiRNAs) targeting viral sequences (Duan *et al.*, 2008; Niu *et al.*, 2006). It has been used in grapevine against the Nepovirus *Grapevine fanleaf virus*, the aetiological agent of fanleaf degeneration disease (Jelly *et al.*, 2012). Two amiRNAs targeting the *coat protein* gene of the virus were studied by transient expression. The processing of the amiRNA precursor by the plant machinery could be assessed in cocultivated somatic embryos of Chardonnay. Moreover, cotransformation assays involving amiRNA constructs together with GUS sensors provided evidence for *in vivo* recognition and cleavage of the short viral target in the sensor construct. At the same time, two amiRNAs targeting *Grapevine virus A* were developed, based on the precursor of *V. vinifera* miR166f, leading to various levels of resistance in *N. benthamiana* (Roumi *et al.*, 2012).

## Investigation of abiotic stress tolerance

There have long been concerns about the adaptation of grapevine to environmental cues such as desiccation, cold, low-light conditions or soil salinity. However, few functional studies through transient expression of genes involved in such characteristics have yet been reported.

In pioneer experiments, Torregrosa *et al.* (2002) and Verriès *et al.* (2004) studied grapevine *Adh* genes by transient expression in Cabernet Sauvignon cell suspension cultures. The grapevine *Adh* gene family comprises three isogenes expressed in berries that are involved in response to environmental stress. Transcriptional fusion constructs involving *luc* fused to partial sequences of *VvAdh1*, and *VvAdh2* promoters were transferred to grapevine

cells by biolistics. These studies allowed identifying anaerobiose-responsive and ethylene-responsive elements (ARE and ERE) in *Adh* promoters.

An interesting application of transient expression assays was conducted for highlighting *in vivo* protein–protein interaction and its subcellular localization in Cabernet Sauvignon protoplasts. Using a bimolecular fluorescence complementation (BiFC) technique, Saumonneau *et al.* (2008) demonstrated the interaction of a drought response element binding (DREB) protein with the abscisic acid, stress and ripening (ASR) TF MSA that regulates the expression of a glucose transporter. MSA and DREB were produced as fusion proteins to release YFP fluorescence upon heterodimerization. The exclusive localization of heterodimers in the nucleus could be determined at the same time. More recently, the promoter of this *VvMSA* gene could be precisely analysed by fusion of deleted versions of its sequence to *luc* and expression in PEG-transformed protoplasts (Saumonneau *et al.*, 2012).

### Analysis of new promoters

Only few strong constitutive promoters are available for transgene expression in grapevine. Synthetic promoters can be obtained by combining existing core promoters and enhancers. In addition, the future development of cisgenesis could benefit from the identification of endogenous promoters (Gray *et al.*, 2014; Holme *et al.*, 2013).

#### *Synthetic promoters*

In an attempt to engineer new strong constitutive promoters, a series of promoter constructs was investigated by transient expression assays in Thompson seedless somatic embryos (Li *et al.*, 2001, 2004, 2011). At first, the CaMV35S and the *Cassava vein mosaic virus* (CsVMV) promoters as well as enhanced double versions of these sequences and an *Arabidopsis* actin promoter (ACT2) were fused to *gfp* for monitoring their ability to induce gene expression (Li *et al.*, 2001). Whereas the ACT2 promoter failed to induce sufficient reporter gene expression, the enhanced double versions of the CaMV35S and CsVMV promoters induced high levels of GFP fluorescence. Furthermore, various bidirectional dual promoter (BDDP) constructs were investigated in similar transient expression assays involving GUS, GFP or anthocyanin monitoring (Li *et al.*, 2004 and Li *et al.*, 2011). Based on either CaMV35S or CsVMV core sequences coupled with enhancers fragments derived from these two promoters, BDDPs comprised two core promoters in inverted orientations. Interestingly, these engineered promoters were shown to express large amounts of the reporter transcript, compared to tandem configurations for which almost no expression was observed. Moreover, additional enhancer sequences in these BDDP constructs displayed even higher levels of reporter expression.

#### *Grapevine promoters*

Recently, the activity of more than thirty grapevine promoters was analysed using cocultivated somatic embryos and *VvMybA1* as a nondestructive reporter (Li *et al.*, 2012). These promoters are mostly derived from *ubiquitin*, *PR1* and *phenylalanine ammonia-lyase* (*PAL*) genes. At least three ubiquitin promoters induced constitutive expression of the reporter, with levels comparable to the double CaMV35S. In addition, some PR1 and PAL promoters appeared to be strong inducible promoters, showing higher expression levels than previously reported inducible promoters.

## Conclusion and future prospects

A major advantage of transient expression assays is their rapid nature. Indeed, expression can be detected as little as 2–3 days after gene transfer, avoiding the lengthy process of stable transformation, and allowing large-scale genetic analyses. In the last 20 years, transient expression assays enabled the validation of many plant gene functions, as well as promoter activity and transgene functionality, especially in model species like *N. benthamiana* (Goodin *et al.*, 2008). Recently, as described above, transient expression assays have also become a key technology for better understanding grapevine biology. The availability of whole-genome sequences (Di Genova *et al.*, 2014; Jaillon *et al.*, 2007; Velasco *et al.*, 2007) and the recent release of many other sequencing data sets accelerated the development of such assays in *V. vinifera*. In addition, world-wide grape germplasm collections comprising both cultivated and wild accessions of *Vitis*, such as the 'European Vitis database' of about 27 000 unique accessions (Maul *et al.*, 2012), constitute valuable genetic resources for grapevine genetic improvement. In the coming years, transient expression assays will be essential for retrieving information from these databases and identifying genes of agronomic interest.

### Varied applications and methods

Transient expression assays have already contributed to functional analysis in *V. vinifera* through many applications (Tables 1 and 2). First, the function of newly identified genes could be examined by overexpression or silencing methods, although RNA silencing was rarely reported. Characterization of native promoters and subsequent identification of specific regulatory regions have also been achieved through deletion analysis. Furthermore, cotransformation assays allowed *in vivo* demonstration of interactions between TF proteins and gene promoters and highlighted the formation of protein complexes. In addition, transient expression assays were used to localize transgene products to subcellular compartments.

Different methods have been developed for gene transfer and are now available to grapevine biologists: on the one hand, direct transformation methods, and on the other hand, agro-transformation methods (Table 3). PEG-mediated transformation requires the delicate preparation of protoplasts, while particle bombardment can be achieved using varied organs or cell suspension cultures but necessitates expensive and complex equipment. *Agrobacterium*-mediated transformation methods exploit the efficiency of T-DNA transfer to plant cells. Cocultivation with *Agrobacterium* is a very simple method because it is based on spontaneous infection of plant tissues. It has mainly been used for the transformation of somatic embryos that can eventually regenerate into stably transformed plant lines, which is an advantage of this system. Nevertheless, agro-infiltration methods have been widely adopted in recent years, due to easy preparation of plant material (i.e. whole *in vitro* plantlets or detached leaves). In addition, agro-drenching has been developed for transferring virus-derived vectors.

### A first step towards crop improvement

Transient expression assays have been used for shaping new tools for stable transformation: synthetic promoters (Li *et al.*, 2001, 2004, 2011, 2012), minimal linear cassettes (Sanjurjo *et al.*, 2013; Vidal *et al.*, 2006) or silencing constructs (Bertazzon *et al.*,

**Table 3** Comparison of methods for transient gene expression in grapevine (*Vitis vinifera* L.)

| | Ready availability of plant material (d) | Rapidity and ease of gene transfer method | Low cost | Extent of gene expression |
|---|---|---|---|---|
| Agro-transformation (a) | | | | |
| Syringe infiltration (leaves) | + + + | + + (e) | + + + | + + (few areas) |
| Vacuum infiltration (leaves or whole plantlets) | + + + | + + (e) (adjustment of vacuum pressure/duration) | + + (vacuum pump/jar) | + +/+ + + (whole leaf or plantlet) |
| Drenching (c) (roots) | + + + | + + + (e) | + + + | + + + (whole plant) |
| Coculture (somatic embryos/cell suspension cultures) | +/+ + (embryos difficult to obtain) | + + + (e) | + + + | + +/+ + + (f) (cell clusters/embryo parts) |
| Direct transformation (b) | | | | |
| Biolistics (cell suspension cultures/any organ) | + +/+ + + | + (adjustment of helium pressure/distance) | + (gene gun/metal microparticles) | + +/+ + + (f) (organ parts/cell clusters) |
| PEG treatment (protoplasts) | + (poorly effective) | + + + | + + + | + (single cell) |
| Electroporation (protoplasts) | + (poorly effective) | + (adjustment of voltage/capacitance) | + (electroporator/cuvettes) | + (single cell) |

(a) agro-transformation needs DNA sequence insertion into a Ti-plasmid-based vector; (b) *E. coli*-based vectors can be used for direct gene transfer; (c) for *Agrobacterium*-mediated delivery of viral vectors; (d) up to now, *in vitro* cultivated plant material was used almost exclusively; (e) preliminary subculture of *Agrobacterium*; and (f) possible regeneration of stably transformed plants.

2012; Urso *et al.*, 2013). In parallel, viral vectors adapted to grapevine infection were also developed (Kurth *et al.*, 2012; Muruganantham *et al.*, 2009). In addition to functional analysis, these viral vectors could be used for engineering grapes resistant to pests and diseases (Senthil-Kumar and Mysore, 2011). In the future, new grapevine-infecting viruses could be engineered to give rise to viral vectors. Indeed, other full-length infectious clones have been obtained such as for the *Grapevine virus B* (Saldarelli *et al.*, 2000) or more recently for the satellite RNA of the *Grapevine fanleaf virus* (Lamprecht *et al.*, 2013). When coinoculated with its helper virus, this satellite RNA was shown to be infectious and could be engineered as Gosselé *et al.* (2002) did with the RNA satellite virus of TMV.

Transient expression assays have already proved useful to readily validate the functionality and efficacy of a DNA construct that is planned to be stably introduced in grapevine. Up to now, research mainly focused on genes or sequences that could confer resistance to the many diseases that threaten vineyards. Indeed, elite cultivars of *V. vinifera* are very susceptible to numerous pathogens. Transient overexpression of *VvNPR1* and *VvWRKY1* conferred increased resistance to *Plasmopara viticola* (Le Henanff *et al.*, 2009; Marchive *et al.*, 2013). Overexpression of a synthetic antimicrobial compound was shown to trigger resistance against bacterial pathogens (Visser *et al.*, 2012). RNAi is also a promising approach for engineering virus-resistant plants (Duan *et al.*, 2012). An *Arabidopsis* miRNA precursor gene modified to target the *coat protein* gene of the *Grapevine fanleaf virus* was validated by a cotransformation assay (Jelly *et al.*, 2012). As shown by transient expression in *N. benthamiana*, amiRNA constructs based on grapevine miRNA precursors can also efficiently target grapevine viral sequences (Roumi *et al.*, 2012). Recently, Romon *et al.* (2013) observed that the RNA silencing machinery of grapevine is resistant to low temperatures, contrary to that of herbaceous species such as *N. benthamiana* or *A. thaliana*. The RNAi strategy is therefore relevant for conferring virus resistance in this perennial crop which is often subjected to low temperatures. Furthermore, data strongly support the safety of genetically modified crops using RNA-mediated gene regulation and show that this approach is appropriate, since only noncoding RNAs are expressed, as opposed to proteins that potentially show toxicity or allergenicity (Petrick *et al.*, 2013).

Disease resistance genes identified in wild grapes could also be used to create transgenic grapevines. The wild grape *V. pseudoreticulata* accession Baihe-35-1 that is resistant to main fungal diseases has been investigated for the identification of resistance genes that could be transferred to *V. vinifera*. Some candidate genes have been tested for their ability to enhance resistance of susceptible grapevines to fungal pathogens using transient expression assays (Guan *et al.*, 2011; He *et al.*, 2013; Xu *et al.*, 2014). In addition, overexpression of other defence-related genes identified in this accession, such as *VpEIRP1* (Yu *et al.*, 2013) and *VpERF-2* and *-3* (Zhu *et al.*, 2013), improved the defence response of susceptible genotypes of *V. pseudoreticulata* and other genetically distant plant species including tobacco and *Arabidopsis*. These results could be applied to grapevine genetic improvement.

Recently, global climate change has prompted the investigation of traits involved in plant physiology and the response to environmental cues such as drought or soil salinity. Dubrovina *et al.* (2013) discovered novel calcium-dependent protein kinases known to play a role in the adaptation to abiotic stresses in the wild-growing *V. amurensis*. Additionally, a recent study showed differences in stomatal response to dehydration in *V. riparia*, *V. champinii* and some *V. vinifera* cultivars (Hopper *et al.*, 2014). These two reports highlight the importance of exploiting genetic resources of *Vitis* spp for identifying genes involved in resistance to environmental stress that could be tested by transient overexpression prior to use for grapevine improvement.

 

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